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### Title

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### Permalink

<https://escholarship.org/uc/item/3h67r1k0>

### Journal

Laboratory investigation; a journal of technical methods and pathology, 89(10)

### ISSN

0023-6837

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### Publication Date

2009-10-01

### DOI

10.1038/labinvest.2009.79

Peer reviewed



Published in final edited form as:

Lab Invest. 2009 October ; 89(10): 1076–1083. doi:10.1038/labinvest.2009.79.

## Estrogen treatment decreases matrix metalloproteinase (MMP)-9 in autoimmune demyelinating disease through estrogen receptor alpha (ER $\alpha$ )

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### Abstract

Matrix metalloproteinases (MMPs) play a crucial role in migration of inflammatory cells into the central nervous system (CNS). Levels of MMP-9 are elevated in multiple sclerosis (MS) and predict the occurrence of new active lesions on magnetic resonance imaging (MRI). This translational study aims to determine whether *in vivo* treatment with the pregnancy hormone estradiol affects MMP-9 levels from immune cells in patients with MS and mice with experimental autoimmune encephalomyelitis (EAE). Peripheral blood mononuclear cells (PBMCs) collected from three female MS patients treated with estradiol and splenocytes from EAE mice treated with estradiol, estrogen receptor (ER)  $\alpha$  ligand, ER $\beta$  ligand or vehicle were stimulated *ex vivo* and analyzed for levels of MMP-9. Markers of CNS infiltration were assessed using MRI in patients and immunohistochemistry in mice. Supernatants from PBMCs obtained during estradiol treatment in female MS patients showed significantly decreased MMP-9 compared to pre treatment. Decreases in MMP-9 coincided with a decrease in enhancing lesion volume on MRI. Estradiol treatment of mice with EAE reduced MMP-9 in supernatants from autoantigen stimulated splenocytes, coinciding with decreased CNS infiltration by T cells and monocytes. Experiments with selective ER ligands revealed that this effect was mediated via ER $\alpha$ . In conclusion, estradiol acting via ER $\alpha$  to reduce MMP-9 from immune cells is one mechanism potentially underlying the estradiol-mediated reduction in enhancing lesions in MS and inflammatory lesions in EAE.

### Keywords

Estradiol; Experimental Autoimmune Encephalomyelitis; Leukocyte Transmigration; Multiple Sclerosis

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Matrix metalloproteinases (MMPs), particularly MMP-9, have been implicated in the disruption of the blood-brain barrier (BBB) in MS because the capacity of T cells and

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monocytes to transmigrate into the CNS has been shown to be dependent upon the activity of MMP-9. Levels of MMPs have been found to be upregulated in several inflammatory CNS diseases (1) including MS where autopsy studies have shown that MMP-9 is increased in macrophages and lymphocytes within perivascular cuffs (2). Furthermore, serum and CSF levels of MMP-9 are higher in relapsing remitting multiple sclerosis (RRMS), particularly in patients during relapse and in those with enhancing lesions on MRI (3–6). Increased MMP-9 serum levels have also been found in patients with clinically isolated syndrome and MMP-9 levels further increased in patients who developed clinically definite MS compared to stable levels in patients who did not convert (7).

During pregnancy, relapse rates in MS are decreased by approximately 80% during the last trimester (8), a time when the pregnancy hormone estriol reaches its highest levels. Studies in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, have shown that estriol treatment ameliorates disease (9, 10) and decreases inflammatory lesions in the spinal cord (11). Anti-inflammatory effects of estrogens are complex and include effects on chemokines, cytokines, dendritic cell function, and T regulatory cell subpopulations (12). Estriol has been shown to downregulate TNF $\alpha$  (10), a cytokine known to activate MMP-9 (13). Consistent with the anti-inflammatory effects of estriol in EAE, a recent pilot study of estriol treatment in female MS patients showed decreased levels of TNF $\alpha$  from peripheral blood mononuclear cells (14) and decreases in the number and volume of gadolinium-enhancing lesions on MRI (15). Finally, T cells obtained from MS patients, which were treated *in vitro* with pregnancy levels of estriol showed decreased MMP-9 levels and lower migratory capacity *in vitro* (16). What remains unclear is whether MMP-9 downregulation occurs *in vivo* when estriol is administered at a pregnancy dose and if this is related to decreases in markers of CNS infiltration during autoimmune demyelinating disease. In addition, it is unclear whether this effect is mediated through estrogen receptor (ER)  $\alpha$  or ER $\beta$ .

## Materials and Methods

### Female RRMS patients

Peripheral blood mononuclear cells (PBMCs) were obtained from three premenopausal female patients with clinically definite RRMS who had participated in our pilot trial of oral estriol treatment with 8 mg/day for a duration of 6 months (15). The study was approved by the UCLA Human Subjects Protection Committee, and informed consent was obtained. The dose used has been shown to yield estriol levels in the blood that approximated 6-month pregnancy levels in humans (15). PBMCs were isolated and cryopreserved at three time points: prior to estriol treatment, at the end of the six month estriol treatment period, and 3 months after the cessation of treatment.

### PBMC cultures, MMP measurement and intracellular MMP-9 staining

PBMCs were cultured at  $1 \times 10^5$  per well with PHA (5  $\mu$ g/ml; Sigma-Aldrich, at 37C, 5% CO<sub>2</sub>) and supernatants were harvested after 48h. Culture supernatants were assayed for levels of MMP-9, its inhibitor TIMP-1, MMP-2 and its inhibitor TIMP-2 using SearchLight multiplex assays. To assess functional proteolytic activity of MMP-9 in the PBMC cell

culture supernatants, zymography assays were used. Supernatants were diluted 1/1 in 2× zymogram sample dilution buffer (NOVEX, San Diego, CA). Then, 10 ml of diluted sample was loaded onto a precast 10% Tris/glycine gel with 0.1% gelatin incorporated as substrate (NOVEX). Gels were electrophoresed at 125 V for 90 minutes and then renatured for 30 min in 1× renaturing buffer (NOVEX) at room temperature. This was followed by incubation in 1× developing buffer at 37°C for 18 h. Gels were stained in 0.5% Coomassie blue R-250 (Bio-Rad, Hercules, CA) dissolved in 30% methanol/10% acetic acid and destained in the same solution without dye. Gels were scanned using an Epson 4870 scanner, and converted to grayscale in Adobe Photoshop. Band intensities were quantified by ImageJ software using the semi-automated Gel Analysis Tool.

For intracellular MMP-9 staining, PBMCs were stimulated with 5µg/ml PHA for 24 hours in the presence of monensin (2µM) to allow for intracellular accumulation. Cells were washed and stained with conjugated surface antibodies for CD3 (APC), CD14 (APC), CD16 (PerCp), and CD56 (PE) (Biolegend, San Diego, CA), washed, fixed and permeabilized with Cytofix/Cytoperm solution (BD PharMingen, San Diego, CA). Then, cells were stained with FITC-labeled Ab specific for MMP-9 or isotype control (R&D Systems, Minneapolis, MN), washed and resuspended for FACS analysis on a FACSCalibur instrument (BD Biosciences, San Diego, CA) using CellQuest software (BD Biosciences, San Diego, CA).

### Brain MRI and lesion quantification

MRI scans obtained in RRMS patients in the estriol trial were analyzed at three time points: prior to estriol treatment, in the last month of estriol treatment (month 6) and three months after the cessation of treatment. T1-weighted scans with and without gadolinium were performed on a 1.5T G.E. scanner. The volume of gadolinium-enhancing lesions was determined using a semiautomated threshold based technique (Display; Montreal Neurological Institute) as described (15).

### Animals

Female C57BL/6 mice, 8 weeks of age, were purchased from Tacomix (Germantown, NY). Animals were housed under guidance set by the National Institutes of Health, and experiments were conducted in accordance with the University of California, Los Angeles, Chancellor's Animal Research Committee and the Public Service Policy on Humane Care and Use of Laboratory Animals.

### Active EAE induction

Active EAE induction ensued with subcutaneous injection of an emulsion containing the autoantigen, myelin oligodendrocyte glycoprotein (MOG) peptide, amino acids 35–55 (300µg/mouse) and *Mycobacterium tuberculosis* (500µg/mouse) in complete Freund's adjuvant. Mice were monitored daily for EAE disease severity using the standard EAE grading scale as described (17).

### Treatment and reagents

Gonadally intact female mice were treated with 60 day continuous release estriol pellets (5mg) or placebo pellets (Innovative Research of America, Sarasota FL) beginning 7 days

prior to EAE induction. This dose has previously been shown to yield circulating estriol levels that approximated levels of estriol during late natural pregnancy in a mouse (9).

Additional sets of mice were anesthetized with isoflurane, ovariectomized and allowed to recuperate for 10 days. Animals were then treated with a selective ER $\alpha$  ligand propyl pyrazole triol (PPT) at 10 mg/kg per day, selective ER $\beta$  ligand diarylpropionitrile (DPN) at 8 mg/kg per day, or vehicle beginning 7 days before EAE induction and throughout the entire disease duration by daily s.c. injections. These doses of ER ligands have previously been shown to yield expected effects on a positive control tissue (uterus) (18). On day 35–40 after disease induction, animals were sacrificed, spleens removed and animals perfused for immunohistochemistry as described (18).

### Splenocyte cultures and MMP-9 measurement

Splenocytes were isolated and stimulated *in vitro* with autoantigen, MOG peptide 35–55, at 25 $\mu$ g/ml for 48 hours. MMP-9 protein levels were assayed in culture supernatants using SearchLight multiplex assays (anti-mouse antibodies). MMP-9 amount in the splenocyte culture supernatants was assessed with zymography as described above.

### Immunohistochemistry

Spinal cords were isolated from perfused mice and processed as described (18). Free-floating cross sections (25  $\mu$ m thick) were cut with a sliding microtome and collected serially in PBS. Consecutive sections were examined by immunohistochemistry. The following primary antibodies were used for T cells (CD3, 1:500, BD Pharmingen, San Diego, CA), and macrophages (Mac3, 1:300, BD Pharmingen, San Diego, CA) and a nuclear stain DAPI (2ng/ml; Molecular Probes). Mounted and stained sections were examined and photographed using a confocal microscope (Olympus Spin disc confocal microscope, Japan). To quantify immunostaining results, sections from thoracic spinal cord levels T1–T5 were examined, three from each mouse, with  $n = 3$  mice per treatment group. Cell numbers were quantified (at  $\times 40$  magnification) by counting the CD3+/DAPI+ and Mac3+/DAPI+ positive cells per 100 $\mu$ m<sup>2</sup> in the dorsal column of the spinal cord by a blinded observer.

### Statistical analysis

MMP levels were compared in RRMS patients from pre-treatment to treatment period using paired  $t$  tests. MMP-9 levels between estriol and vehicle treated mice with EAE were compared using independent  $t$  tests. MMP-9 levels in groups of mice treated with ER $\alpha$  ligand, ER $\beta$  ligand or vehicle were compared using one-way ANOVA with Bonferroni-adjusted post tests. Group differences in EAE scores were tested using repeated measures mixed model ANOVA with Bonferroni-adjusted post tests. Group differences in T cell and macrophage counts in immunohistochemistry were tested using one way ANOVA with Newman-Keuls multiple comparison tests. A value of  $p < 0.05$  was considered statistically significant. All analyses were computed using GraphPad Prism Software 4.0 for Macintosh.

## Results

### MMP-9, but not MMP-2, is decreased by estriol treatment in RRMS

MMP-9 levels and activity were decreased in RRMS patients treated with pregnancy levels of estriol. Zymography, an enzymatic activity based assay, showed decreased amounts of MMP-9 in supernatants from *ex vivo* stimulated PBMCs obtained from patients during estriol treatment compared to pre and 3 months post after treatment (Figure 1A). In addition, during treatment, a significant decrease in MMP-9/TIMP-1 ratio ( $p=0.04$ , Figure 1B) was observed in supernatants from PBMC cultures. This was driven by significant decreases in MMP-9 ( $p=0.02$ , Figure 1C), whereas TIMP-1 was not altered ( $p=0.76$ , Figure 1D). No significant changes were observed in MMP-2/TIMP2 ratio ( $p=0.51$ , Figure 1E), MMP-2 ( $p=0.81$ , Figure 1F) or TIMP-2 levels ( $p=0.60$ , Figure 1G).

Within the same three RRMS patients, changes in MMP-9 were accompanied by a simultaneous decrease in enhancing (Gd+) lesion volume during estriol treatment as compared to before treatment (Figure 1H), consistent with our previous publication showing that estriol treatment significantly decreases Gd+ lesions (15). Representative scans from one subject at baseline and during treatment (Figure 1I) show resolution of an enhancing lesion (white arrow). Flow cytometric analysis revealed that T cells were a major source of MMP-9 in PBMCs stimulated with PHA (Figure 1J). This however does not rule out MMP-9 production by non-T cells using other stimulation conditions.

### Estriol treatment decreases MMP-9 in EAE

Next, we ascertained whether we could replicate the observation from the estriol trial in female RRMS patients by administering estriol treatment to female C57BL/6 mice with EAE. Both zymography ( $p=0.04$ ; Figure 2A–B) and protein measurement by SearchLight assays ( $p<0.001$ , Figure 2C) showed decreased MMP-9 in supernatants obtained from autoantigen-stimulated splenocytes of estriol treated EAE mice compared to vehicle treated. Clinically, estriol treatment significantly ameliorated disease severity ( $p<0.0001$ ; Figure 2D).

Immunohistochemistry revealed that estriol treatment decreased infiltration by T cells ( $p=0.001$ ; Figure 2E, representative images shown in Figure 2F and 2G) and macrophages ( $p=0.04$ ; Figure 2E, representative images shown in Figure 2H and 2I) in the spinal cord.

### Decrease in MMP-9 in EAE is mediated via ER $\alpha$

Effects of estrogens are mediated primarily by nuclear receptors ER $\alpha$  and ER $\beta$ , which have distinct tissue distribution and function. Thus, we next sought to determine whether downregulation of MMP-9 by estriol treatment of EAE was mediated via ER $\alpha$  or ER $\beta$ . To eliminate the effects of endogenous estrogens, female mice were ovariectomized in these experiments. Active EAE was induced in female C57BL/6 mice treated with optimal doses of a selective ER ligand for ER $\alpha$  (PPT), ER $\beta$  (DPN), or with vehicle. Zymography revealed decreased MMP-9 in autoantigen-stimulated splenocyte supernatants from ER $\alpha$  ( $p<0.05$ ) but not ER $\beta$  ligand treated EAE mice ( $p>0.05$ ) compared to vehicle treated (Figure 3A–C). This

was confirmed by significant decreases in MMP-9 protein levels in splenocyte supernatant from ER $\alpha$  ( $p<0.01$ ), but not ER $\beta$  ligand ( $p>0.05$ ) treated EAE mice (Figure 3D).

Clinically, ER $\alpha$  ligand treatment was associated with an early and complete abrogation of disease ( $p<0.0001$ , Figure 3E). ER $\beta$  ligand-treated mice, as compared with vehicle-treated mice, were not significantly different early in disease (up to day 20 after disease induction) but then became significantly improved later during EAE ( $p<0.001$ ).

Immunohistochemistry analysis indicated decreased spinal cord infiltration by T cells (Figure 3F, representative images shown in Figure 3G and 3H) and macrophages (Figure 3F, representative images shown in Figure 3I and 3J) in ER $\alpha$  ligand ( $p<0.05$ ) treated but not ER $\beta$  ligand ( $p>0.05$ ) treated mice compared to vehicle treated EAE mice.

## Discussion

In this study, we found that MMP-9 levels were decreased with estriol treatment at pregnancy doses in patients with RRMS. This decrease in MMP-9 coincided with a decrease in enhancing lesions on MRI. We then showed that MMP-9 levels were also decreased with estriol treatment at pregnancy doses in mice with EAE. Finally, we used this model to show that the estriol-mediated decrease in MMP-9 coincided with a decrease in T cell and macrophage infiltration into the CNS and that this decrease was mediated through ER $\alpha$ .

Estrogens have a wide range of effects on the immune system that could be protective in EAE including downregulation of cytokines, chemokines, dendritic cell function, and induction of regulatory T cells (12). Since estrogens are endogenous regulators of a great number of biological functions, a wide range of effects in the immune system is expected and no single mechanism is likely to be solely responsible for the protective effects in autoimmune disease. In the context of MS and EAE, MMP-9 may be of importance because it plays a key role in transmigration of immune cells into the CNS, which is an early step in our current understanding of MS pathogenesis. MMP-9 is selectively expressed on Th1 cells compared to Th2 cells and is responsible for a higher migratory capacity of Th1 cells (19). Human monocytes have also been found to express high levels of MMP-9, which was linked to their migratory capacity in experimental models of the blood brain barrier (20). The importance of MMP-9 in autoimmune inflammation is further underscored by a study demonstrating that GM6001, a matrix metalloproteinase inhibitor, ameliorated the clinical severity of EAE and reduced blood brain barrier disruption (21).

MMP-9 has been shown to be decreased by other immunomodulators such as IFN $\beta$  (22, 23) and minocycline (24). Both drugs are also highly effective in reducing enhancing lesions on MRI by approximately 80% within the first three months of treatment (25, 26). In contrast, there is no evidence to date that glatiramer acetate (GA) affects MMPs (27). Interestingly, GA does not significantly decrease enhancing lesions on serial MRI until month 6–9 of treatment (28). The ability of a drug to decrease MMP-9 thus appears to be linked its ability to mediate an early and robust reduction in enhancing lesions. Our previous report demonstrating that estriol can decrease enhancing lesions by approximately 80% (15) is consistent with our finding herein that estriol treatment reduces MMP-9.



The synthetic estrogen ethinyl estradiol has been shown to decrease TNF $\alpha$  as well as MMP-9 activity both in the peripheral immune system and the CNS in EAE (29), which is in line with our observation of decreased MMP-9 during treatment with pregnancy doses of estriol. Studies using ER $\alpha$  signaling deficient mouse strains have demonstrated that clinical protection from EAE by estradiol (30) and estriol (31) depends on signaling through ER $\alpha$ . Correspondingly, anti-inflammatory mechanisms of estrogens have been found to be mediated by ER $\alpha$ . We have previously reported that treatment with a selective ER $\alpha$  ligand, but not with an ER $\beta$  ligand, has immunomodulatory effects on peripheral cytokine production and reduces CNS infiltration (18). In addition to these peripheral immune effects, Garidou et al. (32) have elegantly shown that ER $\alpha$ -mediated regulation of resident CNS cells is important for amelioration of EAE. In another study, estradiol decreased MMP-9 production by microglia in ER $\beta$  KO, but not ER $\alpha$  KO, mice in a model of non-autoimmune CNS inflammation (33). Together, these results and our findings suggest that estrogens may interfere with CNS infiltration by inhibiting MMP-9 both within the periphery and the CNS in an ER $\alpha$ -dependent manner.

Interestingly, while ER $\alpha$  ligand treatment abrogated EAE at the onset and throughout the disease course, ER $\beta$  ligand treatment had no effect at disease onset but promoted recovery during the chronic phase of the disease. The late improvement of disability in ER $\beta$  treated mice is in accordance with our previously published study (18). There, we showed that while ER $\beta$  ligand treatment had no effects on splenocyte cytokine production and CNS inflammation, it reduced demyelination and preserved axon numbers in white matter, as well as decreased neuronal abnormalities in gray matter. This suggests a directly neuroprotective mechanism of ER $\beta$  agonists independent of anti-inflammatory effects. These findings are in line with other recent studies using transgenic mice (34) and selective ER $\beta$  agonists (35), indicating that the beneficial effects of estrogen on cognitive function are dependent on the ER $\beta$  pathway. Selective ER $\beta$  agonist effects on cognition have been linked to increased dendritic branching and upregulation of key synaptic proteins including PSD-95, synaptophysin, and AMPA-receptor subunit GluR1 in the hippocampus (36). To date, the exact mechanisms how ER $\beta$  neuroprotection in EAE occurs however remain unclear and are the subject of ongoing studies.

Matrix metalloproteinases, and most importantly MMP-9, play a central role for term labor when expression and activation of MMP-9 increases during parturition (37). Elevated levels of MMP-9 have been implicated in several preterm perinatal complications including spontaneous preterm labor, premature rupture of fetal membranes, and preeclampsia (37). Thus, MMP activity is tightly regulated during pregnancy. MS patients as well as individuals with other inflammatory autoimmune diseases such as rheumatoid arthritis (RA), uveitis, and psoriasis experience clinical improvement during pregnancy, with a temporary 'rebound' exacerbation postpartum (8, 38–43). MMP-9 could represent a common mechanism for immune cell homing in all of these disorders regardless of the target tissue of the autoimmune attack since levels of MMP-9 have been found to be elevated in RA (44), psoriasis (44), and uveitis (45, 46), in particular in patients with active disease. In psoriasis, effective therapy with anti-TNF $\alpha$  was associated with decreased levels of MMP-9 in serum and skin lesions (47). These findings suggest that MMP-9 downregulation may be a shared



mechanism that could underlie the decrease in disease activity of inflammatory autoimmune disorders such as MS, RA, uveitis, and psoriasis during pregnancy.

This paper has focused on a potential beneficial effect of downregulation of MMP-9 during estriol treatment to decrease immune cell infiltration into the target tissue in autoimmune disease, however other effects of MMP-9 that could play a role in MS pathology should also be considered. For example, high levels of MMPs in the CNS can contribute to demyelination and toxicity to axons (48). On the other hand, there is also emerging evidence that MMPs may be important for mediation of tissue repair (1). The regulation of these multiple and complex mechanisms during pregnancy and estriol treatment warrants further investigation.

## Acknowledgements

The authors would like to thank Dr Richard Olmstead for assistance with the statistical analyses.

Supported by the NIH (RO1 NS45443) and the National Multiple Sclerosis Society (grants RD3407, CA 1028, and FG 1702-A-1)

## Abbreviations

<b>BBB</b>	blood brain barrier
<b>CNS</b>	central nervous system
<b>DPN</b>	diarylpropionitrile
<b>EAE</b>	experimental autoimmune encephalomyelitis
<b>ER</b>	estrogen receptor
<b>MMP</b>	matrix metalloproteinases
<b>MOG</b>	myelin oligodendrocyte glycoprotein
<b>MS</b>	multiple sclerosis
<b>MRI</b>	magnetic resonance imaging
<b>PBMC</b>	peripheral blood mononuclear cell
<b>PPT</b>	propyl pyrazole triol
<b>RRMS</b>	relapsing-remitting MS
<b>TIMP</b>	tissue inhibitor of metalloproteinases

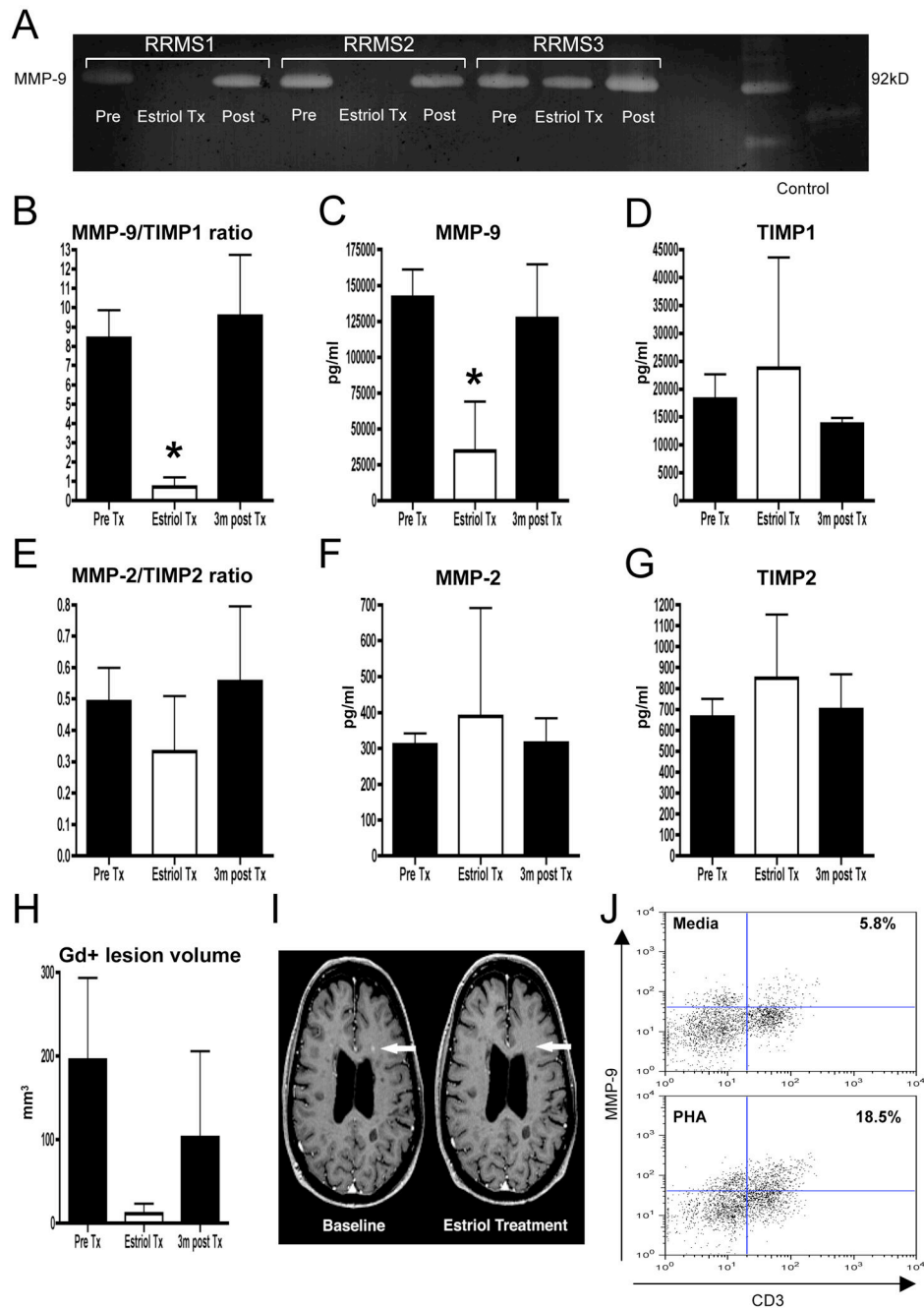
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**Figure 1. MMP-9 regulation by estradiol in multiple sclerosis**

Six month of estradiol treatment reduced MMP-9 bioactivity in peripheral blood mononuclear cell (PBMC) culture supernatants (PHA stimulated) as measured by zymography compared to pre-treatment (pre) and 3 month after treatment cessation (post) in three female patients with relapsing-remitting MS (**A**). Estradiol significantly decreased MMP-9/TIMP1 ratio (**B**). This effect was driven by decreases in MMP-9 levels (**C**), while estradiol had no effect on TIMP1 (**D**). No significant changes were observed on MMP-2/TIMP2 ratio (**E**), MMP-2 (**F**), or TIMP2 (**G**). Decreased volumes of gadolinium-enhancing lesions on MRI occurred

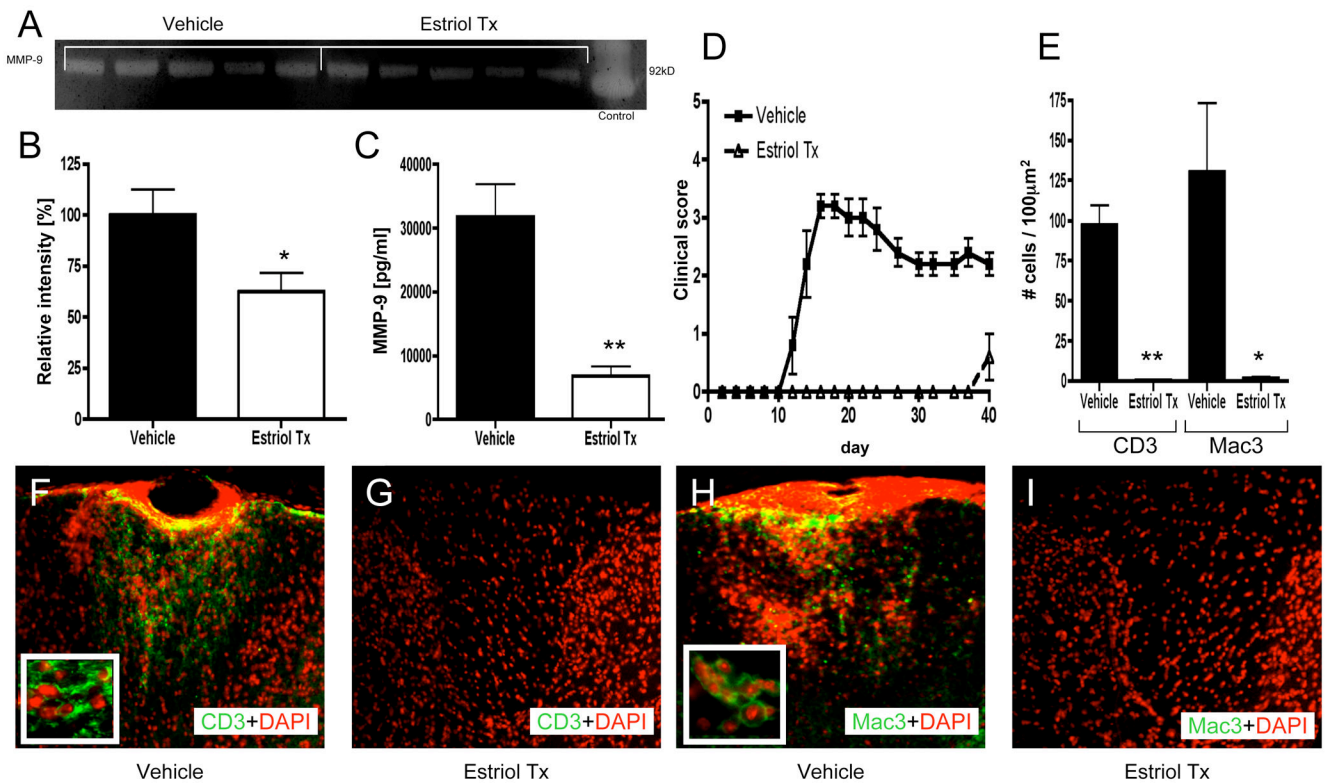
during estriol treatment (**H**). Representative scans from one subject show resolution of an enhancing lesion (**I**, white arrow). Intracellular staining using flow cytometry indicated that T cells (upper right quadrant) were a major source of MMP-9 in PHA activated PBMCs (**J**).

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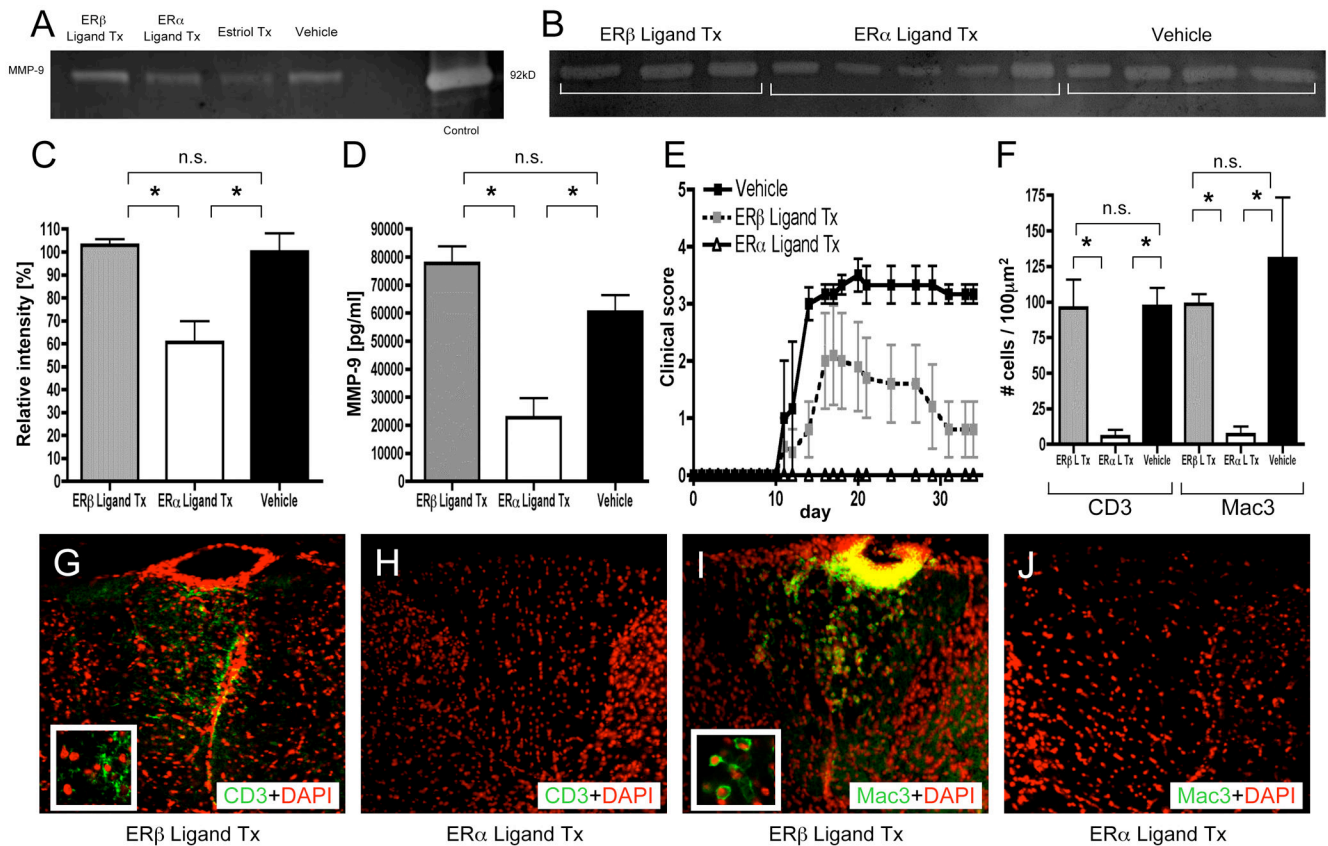
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**Figure 2. Estriol regulation of MMP-9 in active experimental autoimmune encephalomyelitis (EAE)**

Autoantigen-stimulated splenocyte cultured supernatants from estriol treated EAE mice showed decreased MMP-9 bioactivity (A, quantification in panel B) as measured by zymography. Band intensity measurements were normalized by setting the mean band intensity of the vehicle treated group as 100%. Values are expressed as relative intensity (%) for all animals. Estriol treated animals also showed significantly reduced MMP-9 protein levels (C) compared to vehicle treated (n=5 in each group). This was accompanied by abrogation of clinical disease (D). Estriol treatment decreased infiltration into the CNS by T cells (E). Representative images are shown in panels F–G (T cells are labeled with anti-CD3 antibodies, green staining). Similarly, estriol treatment decreased CNS infiltration by macrophages (E). Representative images are shown in panels H–I (macrophages are labeled with anti-Mac3 Gold antibodies, green staining). The nuclear stain DAPI (pseudocolored red) was used to identify all cell nuclei. Images show dorsal column of spinal cord at  $\times 10$  magnification (insets at  $\times 40$ ).





**Figure 3. Estriol-induced MMP-9 downregulation in EAE is mediated through ERα**

Decreased bioactivity of MMP-9 in supernatants from splenocyte cultures compared to vehicle treated was seen in ERα ligand and estriol treated mice, but not in vehicle or ERβ ligand treated mice (**A** pooled supernatants from 4 animals in each group, **B** supernatants from individual animals, **C** quantification). Band intensity measurements were normalized by setting the mean band intensity of the vehicle treated group as 100%. Values are expressed as relative intensity (%) for all animals. Significantly decreased MMP-9 protein levels were observed in ERα ligand treated EAE mice (n=5) compared to vehicle treated EAE (n=4), while there was no difference between vehicle treated and ERβ ligand (n=3) treated mice (**D**). ERα treatment completely abrogated clinical disease (**E**). ERα, but not ERβ, ligand treatment reduced infiltration into the CNS by T cells (**F**). Representative images are shown in panels **G–H** (T cells are labeled with anti-CD3 antibodies, green staining). Similarly, ERα, but not ERβ, ligand treatment decreased macrophages infiltration (**F**). Representative images are shown in panels **I–J** (macrophages are labeled with anti-Mac3 antibodies, green staining). The nuclear stain DAPI (pseudocolored red) was used to identify all cell nuclei. Images show dorsal column of spinal cord at ×10 magnification (insets at ×40).